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Clearance of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) and immunological changes in experimentally injected *Macrobrachium rosenbergii*

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ABSTRACT

Macrobrachium rosenbergii was experimentally challenged with *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) to study the clearance of these viruses and consequent changes in various immunological parameters. The healthy animals were injected *Mr*NV and XSV intramuscularly and various organ samples such as gill tissue, head soft tissue, pleopods and intestine were collected at different time intervals of 3, 5, 10, 15, 25, 50, 75 and 100 d post-infection (p.i.) to study the viral clearance. Tissue tropism and clearing of *Mr*NV and XSV were confirmed by RT-PCR, nested RT-PCR and bioassay. These 2 viruses failed to cause mortality or clinical signs of disease in injected adult prawns during the experimental period of 100 days. The result of RT-PCR analysis revealed that all the organs showed positive for both viruses by single step RT-PCR on 3, 5 and 10 d p.i., positive by nested RT-PCR on 15 and 20 d p.i. and all the organs became negative at 25 d p.i. onwards. The viral inoculum prepared from the tissue of *Mr*NV and XSV-injected *M. rosenbergii* at 3, 5, 10, 15 and 20 d p.i. caused 100% mortality in post-larvae of *M. rosenbergii* at 9, 8, 7, 10 and 10 d p.i., respectively whereas the inoculum prepared at 25, 50 and 100 d p.i. failed to cause significant mortality in post-larvae of prawn. Immunological parameters such as proPO, superoxide anion, SOD, THC, clotting time and oxyhemocyanin were determined in *Mr*NV and XSV-injected prawns and significant differences in some of the immunological parameters were found in the early days p.i. and became insignificant in the later days p.i.

1. Introduction

Fresh water prawn (*Macrobrachium rosenbergii*) is an economically important crustacean, being cultured large-scale in different parts of the world including India. Generally, *M. rosenbergii* is considered to be a moderately disease-resistant species when compared to penaeid shrimp. No serious disease has been reported so far in prawns except white tail disease (WTD) caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). The WTD was reported for the first time in the island of Guadeloupe in 1995 [1]. It was next reported in Martinique, French West Indies (1999), later in China [2], India [3], recently in Thailand [4] and very recently in Australia [5] and Taiwan [6]. These viruses also spread to other prawn-growing countries gradually. *Mr*NV is a small, icosahedral, non-enveloped virus with a diameter of 26–27 nm and containing the genome of two pieces of ssRNA (RNA1 with size of 2.9 and RNA2 of 1.26 kb) with a single polypeptide of 43 kDa in the capsid. XSV is also an icosahedral virus with a diameter of 15 nm and containing a linear single stranded positive-sense RNA encoding for a capsid protein of 17 kDa.

The previous studies on experimental transmission of *Mr*NV and XSV revealed that these viruses are responsible for high mortality in post-larvae and implicated one or both of these viruses as being responsible for WTD in *M. rosenbergii* [2,7]. But these viruses failed to cause mortality and clinical signs of WTD in intramuscularly injected adult prawns [7]. The RT-PCR analysis showed the occurrence of both viruses in gill tissue, head muscle, stomach, intestine, heart, hemolymph, pleopods, ovaries and tail muscle of experimentally injected adult prawns [7]. The adult of *M. rosenbergii* showed resistance to *Mr*NV and XSV in spite of the presence of these viruses in different organs and the exact mechanism of resistance is not known. A similar type of resistance against white spot syndrome virus (WSSV) has been reported in adult *M. rosenbergii* [8], although the larvae can suffer mortality from it [9] as observed in the case of WTD [3].

Sarathi et al. [10] conducted a time course experimental infection in *M. rosenbergii* challenged with WSSV to examine its clearance and studied the consequent immunological changes.

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The results of PCR and bioassay tests revealed the clearance of WSSV from all the organs in WSSV-challenged *M. rosenbergii* after 75 days post-injection (p.i.) [10]. Pathogen clearance study using *Aeromonas hydrophila* and *Lactococcus garvieae* was carried out to examine the clearance efficiency in *M. rosenbergii* [11–13]. All the above studies indicate the presence of strong clearance efficiency and resistance against viral and bacterial pathogens in *M. rosenbergii*. The exact mechanism by which *M. rosenbergii* exhibits tolerance to these pathogens is still unknown. The aim of the present study was to examine the clearance efficiency of *M. rosenbergii* to clear *Mr*NV and XSV, and determine the various immunological parameters.

2. Materials and methods

2.1. Preparation of viral inocula

Naturally WTD-infected post-larvae (PL) with prominent signs of whitish muscle in the abdominal region were collected from hatcheries located near Nellore, Andhra Pradesh, India and used as the source of viral inoculum for infectivity experiments. Frozen infected PL were thawed and homogenized in a sterile homogenizer, and a 10% (w/v) suspension was made with TN buffer (20 mM Tris–HCl and 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at 4000 × g for 20 min at 4 °C and its supernatant was recentrifuged at 10 000 × g for 20 min at 4 °C. The final supernatant was filtered through a 0.22 μm pore membrane. Then, the presence of *Mr*NV and XSV in tissue suspension was confirmed by RT-PCR using the published primers for *Mr*NV and XSV [3]. The filtrate was then stored at −20 °C for experimental infection.

2.2. Collection and maintenance of experimental animals

Healthy adult prawns, *M. rosenbergii* (30–50 g body weight) were collected from grow-out ponds located near Nellore, Andhra Pradesh and transported to the laboratory. They were maintained in 1000 l fiberglass tanks with continuous aeration at room temperature (27–30 °C) in freshwater. The animals were fed twice a day with commercial pellet feed (CP Prawn feed, Bangkok, Thailand). Healthy post-larvae of *M. rosenbergii* were obtained from a hatchery in a locality in Tamil Nadu with no record of WTD. They were randomly sampled and screened for WTD by RT-PCR assay using published primers [3] prior to infectivity experiments. After collection, the post-larvae were washed with sterile freshwater to remove food and other materials adhering to the body. The washed post-larvae were maintained in glass aquaria (25 l) containing aerated freshwater at a temperature of 27–30 °C and fed twice a day with *Artemia nauplii*.

2.3. Experimental infection

For experimental infection, adult *M. rosenbergii* (10 per group per tank) were maintained in 100-l glass aquarium tanks at room temperature (27–30 °C) with freshwater. The animals were injected intramuscularly in the third abdominal segment with *Mr*NV and XSV inoculum (100 μl per animal) prepared from WTD-infected post-larvae of prawn using a 1-ml insulin syringe. Control prawns were injected with tissue suspension prepared from healthy prawns. Hemolymph samples were randomly collected from experimental animals (3 animals per time point) at 1, 3, 5, 10, 25, 50, 75 and 100 days post-injection for the analysis of immunological parameters. One millilitre of hemolymph was drawn directly from the heart of experimentally *Mr*NV and XSV-injected prawns by inserting a 23 gauge needle attached to a 2 ml syringe containing 1 ml of ice-cold anticoagulant Alsever solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 4.6 at 28 °C). The low pH and EDTA in Alsever's solution functioned as an anticoagulant, also preventing degranulation and cell lysis in crustacean hemocytes [14]. Hemolymph samples were also collected from the control prawns for the immunological assays and the results obtained were compared with the experimental prawns. Target organs such as gill tissue, pleopods, head soft tissue and intestine were dissected out and stored at −80 °C for RT-PCR and bioassay. All the experiments were carried out in triplicate for statistical analysis.

2.4. Clearing of MrNV and XSV in MrNV and XSV-injected M. rosenbergii

RT-PCR and nested RT-PCR assays were carried out to examine the clearance of *Mr*NV and XSV in *Mr*NV and XSV-injected prawns using the primers designed by Sahul Hameed et al. [3]. Bioassay was also carried out for further confirmation of clearing of these viruses in injected prawns.

2.5. RT-PCR and nested RT-PCR for MrNV and XSV

For extraction of total RNA, 150 mg of pieces of different organs (gill tissue, head muscle, intestine and pleopods) from adult prawns (3 animals per time point at 1, 3, 5, 10, 25, 50, 75 and 100 days post-injection) were homogenized in 300 μl of TN buffer. The homogenate was centrifuged at 12 000 × *g* for 15 min at 4 °C and the supernatant was collected, and referred to as crude tissue extract. Total RNA was extracted using TRIzol reagent (GIBCO-BRL) according to the protocol of the manufacturer. Briefly, 1 ml of TRIzol reagent was added to 150 μl of crude tissue extract and mixed thoroughly. After 5 min of incubation at room temperature, 0.2 ml of chloroform was added. The sample was vigorously shaken for 2–3 min at room temperature then centrifuged at 12 000 × *g* for 15 min at room temperature. RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol and dissolved in 50 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). The amount of nucleic acid in the sample was quantified by measuring the absorbance at 260 nm. The purity of the preparation was checked by measuring the ratio of optical density OD_{260nm}/OD_{280nm}. Reverse transcriptase-polymerase chain reaction was carried out using the Reverse-IT™ 1-step RT-PCR kit (ABgene Fairport, NY, USA), allowing RT and amplification to be performed in a single reaction tube. Nested RT-PCR (nRT-PCR) was carried out using the products of RT-PCR wherever it was necessary to confirm the clearance of the viruses. The published primers were used for RT-PCR and nRT-PCR [3,15]. Reactions were performed in 50 μl RT-PCR buffer containing 20 pmol of each primer and RNA template, using the following steps: RT at 52 °C for 30 min; denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s and elongation at 68 °C for 1 min, ending with an additional elongation step for 10 min at 68 °C. For nRT-PCR, reactions were performed in a 20 μl reaction mixture containing 2 μl RT-PCR product, 1 μM of each internal primer (*Mr*NV or XSV), 200 μM deoxynucleotide triphosphate and 1.25 U *Taq* DNA polymerase in PCR buffer supplied with a commercially available kit (Finnzymes, Espoo, Finland). The nRT-PCR protocol for both viruses comprised 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C with a final extension of 10 min at 72 °C. The RT-PCR and nRT-PCR products (10 μl) were then analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.